ELSEVIER

FEMS Microbiology Ecology 24 (1997) 159-172

FEMS MICROBIOLOGY ECOLOGY

Cell-surface hydrophobicity and cell-surface charge of *Azospirillum* spp.

Thelma Castellanos [a], Felipe Ascencio [b], Yoav Bashan [a *]

[a] *Department of Microbiology, The Center for Biological Research of the Northwest (CIB), A. P. 128, La Paz, Baja California Sur 23000, Mexico*

[b] *Department of Marine Pathology, The Center far Biological Research of the Northwest (CIB), La Paz, Baja California Sur 23000, Mexico*

Received 20 April 1997; revised 24 June 1997; accepted 24 June 1997

Abstract

The cell-surface charge and cell-surface hydrophobicity of 10 strains of *Azospirillum* spp. were evaluated by the aqueous, two-polymer phase partitioning assay. *Azospirillum* spp. had moderate cell-surface hydrophobicity and charge, lower than known values for human pathogens. The hydrophobicity and charge values were higher in solid than in liquid media. A general pattern of modifying hydrophobicity and charge by external treatments of the cells of all strains was not found, but various chemicals, temperatures, and enzymatic treatments changed the cell-surface hydrophobicity and charge in different strains. A similar hydrophobic protein was isolated, and partially characterized, from two strains of *A. brasilense* and one strain of *A. lipoferum*. This study proposes: (i) cell-surface hydrophobicity and charge of *Azospirillum* spp. can be affected by external treatments of the bacterium cell, and (ii) hydrophobicity and charge may play a role, perhaps small, in the primary adsorption of *Azospirillum* spp. to surfaces.

Keywords: *Azospirillum*; Cell-surface charge; Cell-surface hydrophobicity; Hydrophobic membrane protein; Plant growth-promoting rhizobacteria

1. Introduction

Azospirillum spp. are plant growth-promoting bacteria associated with economically important crop plants [1,2]. As an almost obligatory first step of the plant and bacterium interaction, the roots have to be colonized. The close proximity of the bacterium and the plant root cells determined, in most cases, the success of positive plant response to inoculation [3]. Root colonization in situ by *Azospirillum* spp. required live bacteria [4], was governed by bacterial motility in the soil, and was stimulated by plant exudates [4-7].

Attachment processes of *Azospirillum* spp. were defined to roots [8-13], to sand particles [14,15], to plant cell suspension [16] and to themselves within bacterial aggregates [17,18], or within bacterial flocs [19]. Two different phases of attachment of *A. brasilense* were proposed. They are similar to those previously proposed by Marshall [20] for the attachment

* Corresponding author. Tel.: +52 (112) 5-3633 ext. 219; Fax: +52 (112) 5-4710 or 5-3625; E-mail: bashan@cibnor.mx

Abbreviations: CSC, cell-surface charge; CSH, cell-surface hydrophobicity; HIC, hydrophobic interaction chromatography; PGPR, plant growth-promoting rhizobacteria

PII S0168-6496(97)00055-X

of soil bacteria to a soil particle. The primary adsorption phase is fast (reaches a maximum level within 2 h of incubation), weak, and probably governed by bacterial proteinaceous compounds [15]. The second phase (named anchoring) is stronger, takes several hours to form (it begins only after 8 h of incubation), is irreversible, and is probably based on bacterial extracellular surface polysaccharides [13,21]. Although inhibition of the primary adhesion has been observed using protein inhibitors [13,15,22], the precise process remains unexplained.

Cell-surface hydrophobicity and cell-surface charge of bacteria have been recognized as measurable physicochemical variables for evaluating bacterial adhesion to surfaces [23,24]. Few studies have been published on cell-wall properties of *Azospirillum* spp. Previous studies have shown *A. brasilense* Cd can attach in large numbers to hydrophobic polystyrene surfaces [9]. *A. brasilense* Cd was evaluated and was found to be hydrophilic [25]. Recently, Dufrêne and Rouxhet [26] noted the involvement of cell-surface proteins and cell-surface hydrophobicity of *A. brasilense* in the adhesion process.

The aims of this study were: (1) to measure the effect of bacterial growth media (liquid or solid) on the hydrophobicity and cell-surface charges of 10 strains of the genus *Azospirillum;* (2) to measure the effect of chemicals, temperatures, and enzymatic treatments on the possible changes in cell-surface charge and hydrophobicity; and (3) to isolate and partially characterize a hydrophobic protein from the *Azospirillum* spp. cell surface.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Ten *Azospirillum* strains were used in this study: *A. brasilense* Sp-245 (donated by J. Döbereiner, CNPBS, Rio de Janeiro, Brazil), Cd (DSM 7030, Braunschweig, Germany), Sp-6 (our laboratory collection), *A. lipoferum* 37 and 779 (donated by E. Ruckdäschel and W. Klingmüller, University of Bayreuth, Germany), *A. lipoferum* JA2 and JA4 (donated by V.L.D. Baldani, CNPBS, Rio de Janeiro, Brazil), *A. lipoferum* 1842 (DSM 1842, Braunschweig, Germany), *A. halopraeferens* Au5 (donated by B. Reinhold-Hurek, Max-Planck Institute, Marburg, Germany), and *A. irakense* KBC1 (donated by Dr. P. Kaiser, INRA, Paris, France).

The culture media used were: nutrient broth (NB) (Merck, Darmstadt, Germany), Luria-Bertani medium (LB) (Difco, Detroit, MI, USA) and nitrogen-free, malate minimal medium (OAB) [27,28]. For standardization, cells were grown in 50 ml of medium in a 250-m1 flask and were incubated at 30 ± 1 °C in a rotary shaker at 120 rpm for 20 h (OD_{540} = 1.05 X 10^9 cfu ml^{-1}; exponential phase of growth of *Azospirillum* species). *A. halopraeferens* cultures were supplemented with 0.25 M NaCI. For solid media experiments in Petri dishes, 20 g of agar 1^{-1} (Sigma, St. Louis, MO, USA) was added, and the cultures were incubated for 48 h at 30 ± 1 °C, corresponding to the exponential phase of these types of media.

2.2. Chemical, temperature, and enzymatic treatments of Azospirillum spp. cells

The effect of chemical treatments on cell-surface hydrophobicity (CSH) and cell-surface charge (CSC) was measured using bacteria growing on solid or liquid NB medium for 48 h at 30 ± 1 °C. All colonies were harvested with a microbiological loop from the plate's surface. The bacteria were suspended in 15 mM sodium phosphate buffer supplemented with 15 mM NaCl (PBS) pH 7.6, and the bacterial suspension was adjusted with PBS to an optical density of 1.0 at 540 nm, equivalent to 10^9 cfu ml^{-1}. Aliquots (900 μl) of this bacterial suspension were mixed with 100-μl aliquots containing 75 mM of $NaIO_4$, 75 mM $NaBH_4$, or 0.025 N H_2SO_4. These chemical and bacteria mixtures were incubated at 22 ± 1 °C for 1 h. Bacterial cells were washed with PBS, and suspended in the PBS at the original concentration. Aliquots (100 μl) were taken to determine CSC and CSH as described later. No loss of cell viability was observed.

The temperature treatments were done by incubating 1 ml of 10^9 cfu ml^{-1} bacterial suspensions in constant temperature baths at 100, 80, 60, and 40°C for 1 h. Aliquots (100 μl) were used to determine CSC and CSH.

The enzymatic treatments of bacterial suspensions

were done similarly by adding 100 µl of one of the following to 900 µl of bacterial suspension: 1 mg ml^{-1} of proteinase K (from *Triterachium album*); trypsin (type 1 from bovine pancreas); protease (type XIV from *Streptomyces griseus);* or chymotrypsin (type 1-S from bovine pancreas) (all from Sigma). These suspensions had a final concentration of 100 µg enzyme ml^{-1}, corresponding to the following units enzyme mg^{-1}: proteinase K 15 U mg^{-1}, trypsin 10900 U mg^{-1}, protease 5.5 U mg^{-1}, chymotrypsin 47 U mg^{-1}. Mixtures were incubated at 22°C for 30 min. Aliquots (100 µl) of these solutions were used to determine CSC and CSH as described below. No loss of all viability was observed.

2.3.Cell-surface charge and cell-surface hydrophobicity assays

CSC and CSH were determined by the aqueous, two-polymer phase partitioning assay of Johansson [29]. The chemical composition of each of the two phases of the three systems of the partitioning assay is given in Table 1.

Bacterial partitioning was done by adding 100 µ1 of a bacterial suspension (109 cfu ml^{-1} grown either on agar or in broth media and suspended in PBS) to 0.9 ml of the phase systems, making the volume of the phase system 1 ml. The suspension of the bacteria in the phase system was mixed by gentle shaking. Immediately, 100-µl aliquots were taken and placed in an ELISA plate. The initial numbers of added bacteria were determined by absorbance. The assay tubes were allowed to stand undisturbed for phase separation for 1 h at 22 ± 1 °C. After separation, 100-µl aliquots were taken from the top phase of each of the three systems (I, II, and III), placed in an ELISA plate and absorbance recorded at 540 nm in a Microplate Reader model 3550 (Bio-Rad, Richmond, CA, USA). The bacterial concentration in the PEG-rich top phase of each system were expressed as the percentage of the bacteria originally added.

Differences in CSC and CSH of *Azospirillum* strains grown in broth or solid NB media, under various culture conditions, using different chemical treatments, temperatures, or enzymes were expressed as Δlog G, the partitioning coefficient, which is defined by the equation:

$$\Delta \log G = \log \frac{\text{G value of (system II or system III)}}{\text{G value of system I}}$$

$$\text{where } G = \frac{\text{\% cells in the top phase}}{\text{\% cells in the rest of the system}}$$

As defined by Johansson [29], the partitioning coefficients will depend on the net charge of the bacterial cell surface interacting with the sulfate groups of the phase containing dextran sulfate, and the affinity of the bacteria for the hydrophobic groups of the phase containing PEG-palmitate. Thus, Δlog G values larger than zero indicate a hydrophobic and negatively charged character of the bacterial cell surface. Calculated negative Δlog G values are expressed in this study as zero CSH and CSC.

2.4. Extraction of cell-surface proteins from A. brasilense Cd, Sp-245, and A. lipoferum 1842

Azospirillum spp. cells were harvested either from five solid nutrient agar (NA) plates or from 250 ml NB medium and washed twice with PBS. Cell pellets were resuspended in 10 ml of 6 M urea (Merck, Darmstadt, Germany) for 90 min at 22±1°C [30]. The cell suspensions were centrifuged at 8000 X *g* for 10 min at 10±1°C. The pellet was discarded and the supernatant treated with 80% $(NH_4)_2SO_4$

Table 1
The aqueous two-polymer phase partitioning assay[a] used in this study

	System I	System II	System III
Phase 1	7.13% (w/w) polyethylene glycol (PEG) in 150 mM NaCI	7.13% (w/w) PEG in 150 mM NaCI	6.73% (w/w) PEG+0.4°/ (w/w) PEG-palmitate in 150 mM NaCI
Phase 2	8.75% (w/w) dextran in 150 mM NaCI	8.35% (w/w) dextran+0.4% dextran sulfate in 150 mM NaCI	8.75% (w/w) dextran in 150 mM NaCI
Used for	reference control	cell-surface charge	cell-surface hydrophobicity

[a]According to the method of Johansson [29].

at 4± 1°C overnight. The precipitate formed was dissolved in 1 ml of 2 M $(NH_4)_2SO_4$ and the entire solution was subjected to hydrophobic interaction chromatographic analysis.

2.5. Hydrophobic interaction chromatography (HIC)

Samples (1 ml) of the urea extraction of the cell-associated proteins from *A. brasilense* Cd and *A. lipoferum* 1842 cells were added, separately, to a 6-ml plastic column (120 mm X 8 mm) packed with octyl Sepharose CL-4B (Sigma). Unbound proteins were washed out with 12 ml of 2 M $(NH_4)_2SO_4$. The adsorbed proteins were eluted with a step gradient of decreasing $(NH_4)_2SO_4$ concentrations (1.5, 1.0, 0.5, 0.25, and 0.1 M) and distilled water. An alternative procedure was to elute all the adsorbed proteins with a single application of 12 ml of distilled H20. The chromatographic analysis was done using a Pharmacia Fine Chemical liquid chromatography controller LCC-500 (Pharmacia, Uppsala, Sweden). Fractions of 2 ml were collected by a fraction collector (Fracc-100; Pharmacia) and were dialyzed against 2 1 of 0.01 M ammonium bicarbonate, pH 8.0, for 48 h. at 4± 1°C, with several replacements of the dialysate. The dialyzed fractions were used for electrophoresis or were stored at -40 ± 1°C for further experiments.

2.6. SDS-PAGE

The dialyzed fractions (concentrated by lyophilization and adjusted to 1 mg ml^{-1} with distilled water) and crude extract of cell-associated proteins (obtained as described above) were separated by SDS-PAGE using 12.5% polyacrylamide slab gels according to the method of Laemmli [31].

2.7. Isoelectric focusing

Cell-associated protein samples were subjected to isoelectric focusing on a gel containing 24.25% acrylamide, 0.75% bisacrylamide, 25% glycerol, 0.1% of FMN (50 mg riboflavin-5-phosphate dissolved in 50 ml H_20), 2% ammonium persulfate, 3 µl of tetramethylethylenediamine (0.08°/0) (TEMED), and 0.6 ml ampholyte Bio-lyte 3/10 (2%) (Bio-Rad) [32]. The isoelectric focusing was run initially at 500 V until the current equalled zero. Afterwards, the gel was fixed for 30 min in a mixture containing: 4 g sulfosalicylic acid, 12.5 ml trichloroacetic acid, and 30 ml methanol, made up to 100 ml with distilled water. The gel was stained for 2 h in a mixture containing 27 ml ethanol, 10 ml glacial acetic acid, 0.04 g Coomassie brilliant blue CBR-250, and 0.5 g $CuSO_4$, made up to 100 ml with distilled water. Finally, the gel was destained for 1-2 h, until bands were visible, in a mixture containing 12 ml ethanol and 7 ml glacial acetic acid made up to 100 ml with distilled water [32].

2.8. Internal amino acid sequencing

Protein obtained from HIC was separated by electrophoresis, extracted from the gel by cutting out the band site and sent to the peptide sequence service at Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBM), Strasbourg, France. As the N-terminal was blocked, the protein was digested with porcine trypsin (Promega Corp., Madison, WI, USA) and the peptide of peak 34 was sequenced. The sequence obtained was compared to known bacterial hydrophobic protein using the SwissProt Data Bank.

2.9. Experimental design and statistical analysis

All experiments were run twice from independent cultures and in duplicate. A replicate consisted of two partitioning assays. The data from both experiments were combined and analyzed by one-way analysis of variance (ANOVA) at $P \leq 0.05$.

3. Results

Ten *Azospirillum* spp. strains were evaluated for CSC and CSH. These two variables were determined with bacteria grown in either solid or liquid media. In general the values of charge and hydrophobicity were higher when the bacteria were grown in solid media. The *A. brasilense* strains were more homogeneous in their behavior than the *A. lipoferum* strains tested. *A. halopraeferens* and *A. irakense* did not show charge and hydrophobicity independent of the media used (data not shown).

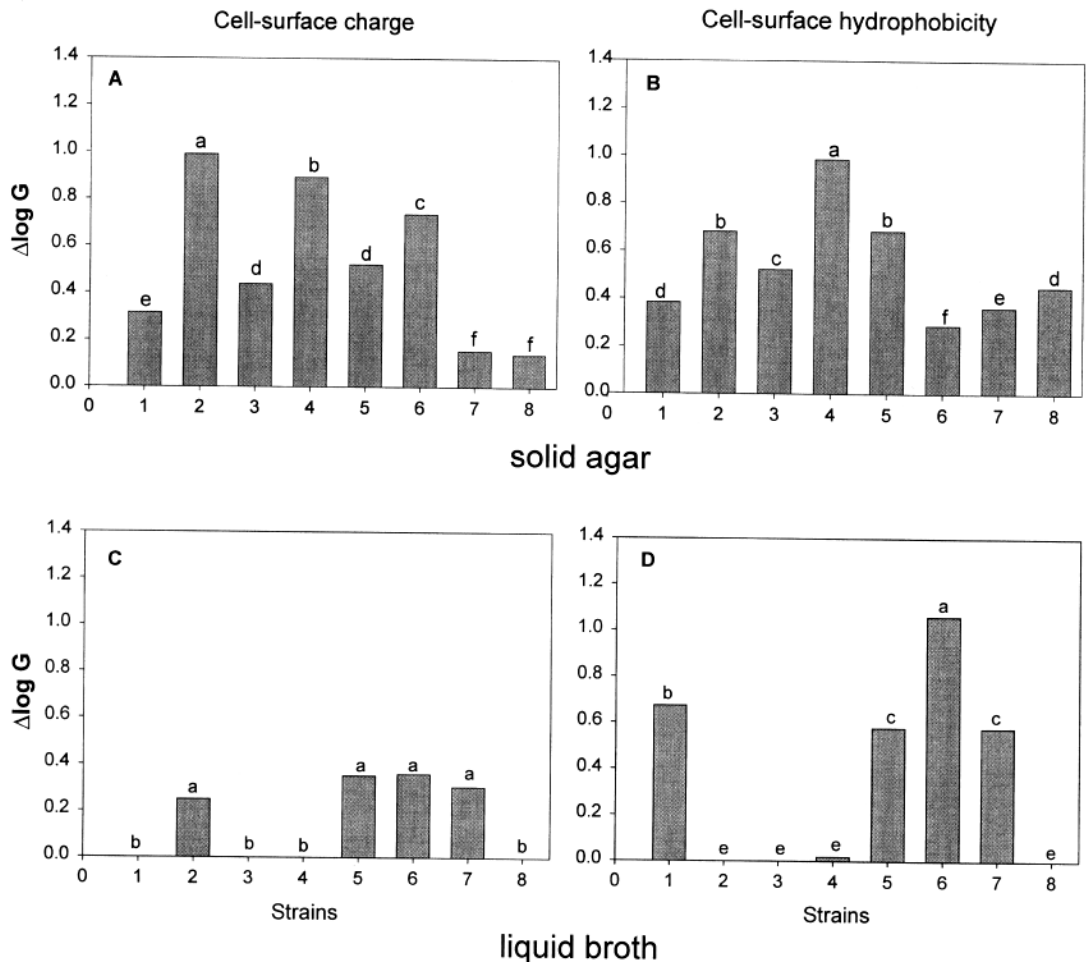

Fig. 1. Effect of solid and liquid media on the CSC (A, C) and CSH (B, D) of eight *Azospirillum* strains: (1) *A. brasilense* Sp-6, (2) *A. brasilense* Sp-245, (3) *A. brasilense* Cd, (4) *A. lipoferum* 37, (5) *A. lipoferum* 779, *(6) A. lipoferum* JA2, (7) *A. lipoferum* JA4, (8) *A. lipoferum* 1842. Statistical analyses for experiments in each medium type were done separately. Each bar represents the mean for two experiments, each done in duplicate. Bars denoted by different letters in each subfigure differ significantly at $P \leq 0.05$.

3.1. Cell-surface charge

The values of the CSC depended on the type of culture media used. When bacteria were grown on solid NA medium, four strains had CSC values lower than 0.5, and four strains had CSC values between 0.5 and 1. *A. brasilense* Sp-245 had the highest CSC value followed by *A. lipoferum* 37 and JA2 (Fig. IA). When bacteria were grown in liquid NB, CSC values were significantly lower. Only four strains had low positive CSC values of 0.2.3, while the rest had a negligible CSC (Fig. 1C). When bacteria were grown on solid LB medium, five strains showed CSC values between 0.5 and 1. One, *A. lipoferum* 37, was slightly lower than 0.5. The other strains had negligible CSC values (data not shown). When the 10 strains were grown on solid OAB medium, five strains showed CSC values between 0.4 and 0.9, and three had negligible values (data not shown).

In liquid LB medium, eight strains had negligible CSC values. Only *A. lipoferum* 37 had low CSC values (data not shown).

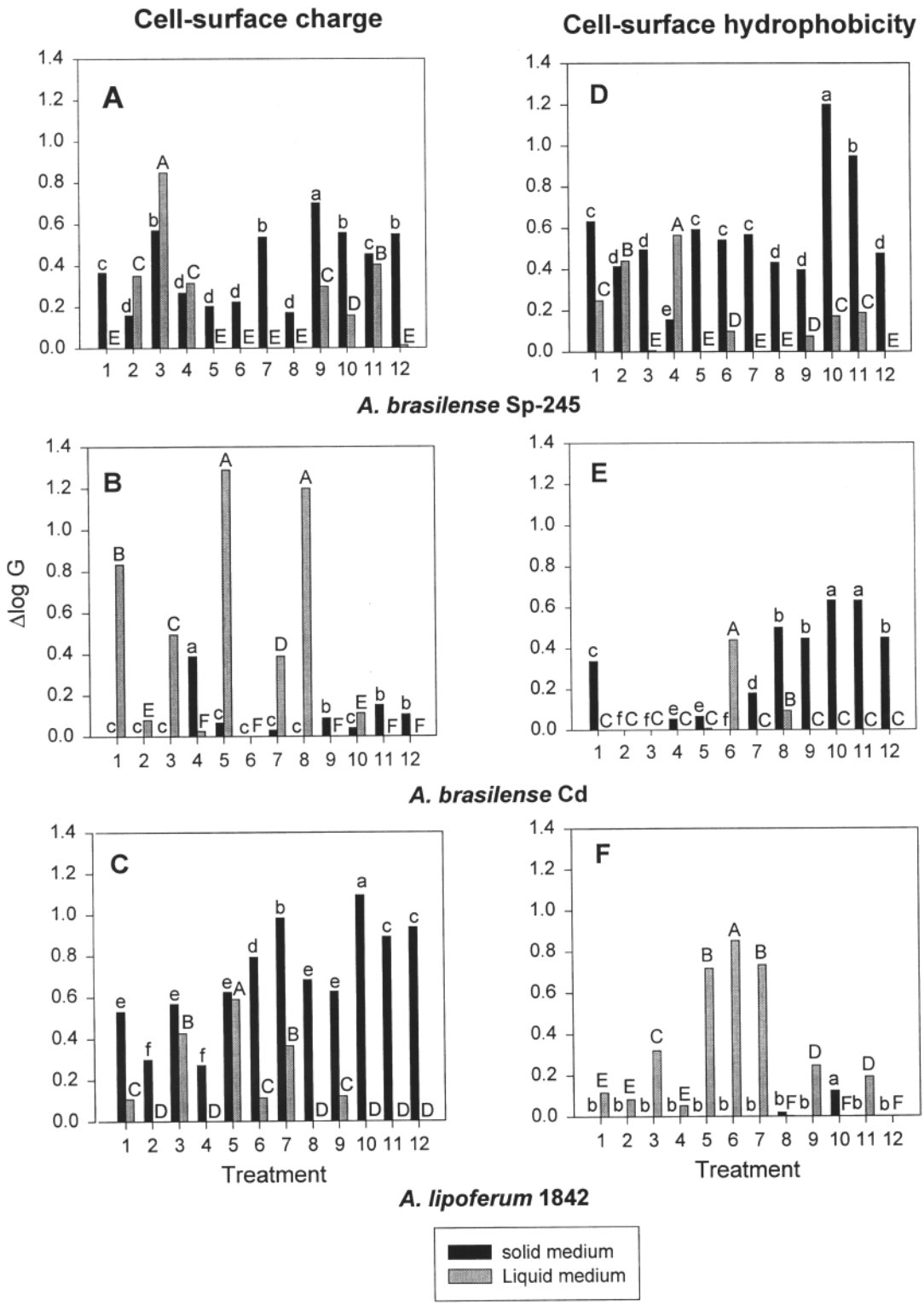
Cell-surface charge
Cell-surface hydrophobicity
A
D
A. brasilense Sp-245
B
E
A. brasilense Cd
C
F
A. lipoferum 1842
Δlog G
Treatment
solid medium
Liquid medium

Fig. 2. Effect of temperature, chemicals, and enzyme treatments on the CSC (A,B,C) and CSH (D,E,F) of *A. brasilense* Sp-245, *A. brasilense* Cd, *A. lipoferum* 1842. Treatments: (1) 40°C, (2) 60°C, (3) 80°C, (4) 100°C, (5) H_2SO_4, (6) $NaIO_4$, (7) $NaBH_4$, (8) protease, (9) proteinase K, (10) trypsin, (11) chymotrypsin, (12) untreated control. Statistical analyses for experiments in each medium type were done separately. Each bar represents the mean for two experiments, each done in duplicate. Bars denoted by a different letter in each subfigure differ significantly at $P \leq 0.05$. Capital letters represent statistics for liquid medium and lower case letters for solid medium.

3.2. Effects of temperature, chemical and enzymatic treatments on CSC

The CSC of three strains, *A. brasilense* Cd, *A. brasilense* Sp-245, and *A. lipoferum* 1842 grown on solid and liquid NB media, was evaluated following different treatments. These treatments were to evaluate whether external treatment of the cells (as those that may occur in the soil) can change the CSC of the cells. On solid medium, when bacteria originally grown at 30°C were subjected to heat treatments (40-100°C), they had similar or lower CSC values compared with cells not heated (Fig. 2A,B,C). Only *A. brasilense* Cd, at the highest temperature tested (boiling, 100°C), increased its CSC (Fig. 2B). None of the three chemical treatments had an effect on the CSC of *A. brasilense* Cd and Sp-245 (Fig. 2A,B). Only NaBH4 increased CSC in *A. lipoferum* 1842 (Fig. 2C). The four enzymatic treatments kept the CSC at positive, but variable, values in all three strains (Fig. 2A,B,C).

In contrast to cells grown on a solid medium, when bacteria grown in liquid media were assayed following the same treatments, the CSC values increased in several of them. The heat treatment at 80°C induced positive CSC values in all three strains tested. Treatment at 40°C enhanced CSC in *A. brasilense* Cd, less in *A. lipoferum* 1842, but not at all in *A. brasilense* Sp-245. Treatments at 100°C enhanced the CSC of only *A. brasilense* Sp-245 (Fig. 2A,B,C). The chemical treatments induced positive CSC values in *A. lipoferum* 1842 and *A. brasilense* Cd but not in *A. brasilense* Sp-245. In *A. brasilense* Cd, the acidic and basic treatments induced positive CSC values, while the oxidative treatment caused no change in values. In *A. lipoferum* 1842, all chemical treatments increased CSC (Fig. 2A,B,C).

The effect of enzymatic treatment differs in the three strains. Enzyme treatment had no effect on *A. lipoferum* 1842, except for proteinase K. In *A. brasilense* Cd, only protease E, and to a lesser extent trypsin, increased CSC. In *A. brasilense* Sp-245, treatment with proteinase K, trypsin, and chymotrypsin, but not protease E, gave increased CSC (Fig. 2A,B,C).

3.3. Cell-surface hydrophobicity

The CSH presented a pattern similar to that of CSC when the bacteria were grown in solid NA (Fig. 1B). Of the cells grown on NA medium, four strains had high CSH, and four medium CSH (Fig. 1B).

On solid LB medium, four strains had high CSH (> 0.5), three strains had medium CSH (< 0.5) and three strains had negligible CSH (data not shown). On OAB solid medium, three strains had high CSH, two medium CSH, and five strains had negligible CSH (data not shown).

When the cells were grown in liquid NB medium, the CSH differed from the CSH on solid media. In liquid NB medium, four strains showed high (> 0.5) CSH, one had very low CSH, and three strains had negligible CSH (Fig. 1D). In liquid LB medium, only two strains, *A. brasilense* Cd and *A. lipoferum* 37, were highly hydrophobic. The other eight strains had low (four strains) or zero (two strains) hydrophobicity. *A. lipoferum* 1842 and *A. irakense* KBC1 were not hydrophobic in either liquid medium (data not shown).

In solid media, the hydrophobicity of three selected strains, *A. brasilense* Cd, *A. brasilense* Sp-245, and *A. lipoferum* 1842, after heat treatment was the same as unheated cells except for *A. brasilense* Sp-245 at 40°C (Fig.2D,E,F). Similarly, chemical treatments did not yield changes in CSH in any strain (Fig. 2D,E,F). Some of the enzymatic treatments s howed positive CSH in *A. brasilense* Cd and in *A. brasilense* Sp-245. The highest values came from treatment with trypsin and chymotrypsin. *A. lipoferum* 1842 showed no CSH values with proteinase K and chymotrypsin but small positive CSH values with protease and trypsin (Fig. 2D,E,F).

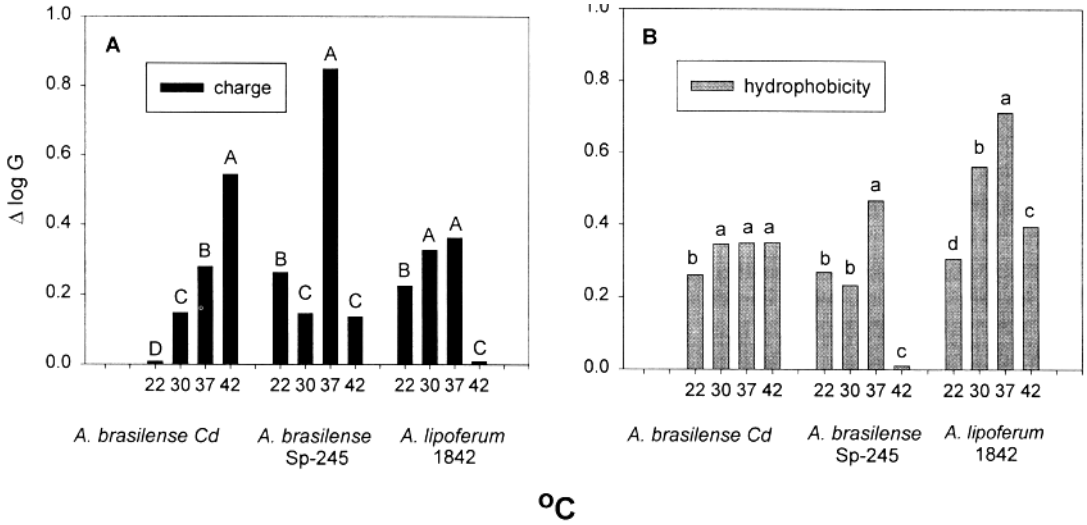

Fig. 3. Effect of different growth temperatures of *A. brasilense* Cd, *A. brasilense* Sp-245 and *A. lipoferum* 1842 on the CSC (A) and CSH (B). Each bar represents the mean of two experiments each done in duplicate. Bars denoted by different letters in each subfigure differ significantly at $P \leq 0.05$.

When the cells were grown in liquid medium NB, heat treatment showed no CSH values for *A. brasilense* Cd, similar to the untreated controls or cells growing on solid medium. *A. brasilense* Sp-245 and *A. lipoferum* 1842 showed CSH values higher than control (Fig. 2D,E,F). After chemical treatments, *A. lipoferum* 1842 showed high CSH values for all the chemicals. The two strains of $_{A}$. *brasilense* showed positive values only with $NaIO_4$. The enzymatic treatments had no effect on the CSH of *A. brasilense* Cd. Low positive values were found for *A. brasilense* Sp-245 using proteinase K, trypsin, and chymotrypsin, and for *A. lipoferum* 1842 with proteinase K and chymotrypsin (Fig. 2D,E,F).

3.4. Effect of bacterial growth temperature on the CSC and CSH

When cells of *A. brasilense* Cd, Sp-245, and *A. lipoferum* 1842 were grown at different temperatures, their CSC varied. In *A. brasilense* Cd, CSC increased from 22 to 42°C (Fig. 3A). In *A. brasilense* Sp-245, the highest CSC was at 37°C. In *A. lipoferum* 1842, CSC values increased with temperature from 22 to 37°C but decreased to zero at 42°C (Fig. 3A). The CSH did not change in *A. brasilense* Cd between 30 and 42°C. *A. brasilense* Sp-245 had positive values from 22 to 37°C and a zero value at 42°C. *A. lipoferum* 1842 showed increased values from 22 to 37°C but a decreased value at 42°C (Fig. 3B).

3.5. Isolation of a cell-surface hydrophobic protein

From the hydrophobic chromatography, six main peaks were obtained for *A. brasilense* Sp-245 and Cd. Each peak corresponds to each of the concentrations of $(NH_4)_2SO_4$ used in the elution. For *A. lipoferum* 1842, the peak corresponding to elution with 1 M of $(NH_4)_2SO_4$ is missing (Fig. 4). When the fractions of these chromatographies were separated in SDS-PAGE, one band was constantly seen in fractions 2-6. Its quantity was greater in the fraction eluted with water, especially for *A. lipoferum* 1842 (Fig. 5).

The molecular mass of this protein was 43 kDa (Fig. 5A). Isoelectric focusing showed three bands, at pH 4.45, 4.7 and at 5.1 (Fig. 6). After trypsin digestion, the internal sequence of peak 34 of this protein was: Ala, Tyr, Ile, Phe, Ala, Gln, Gly, Ser, Phe, Gly, Gln. This is a hydrophobic sequence that does not resemble any known sequence of bacterial hydrophobic protein.

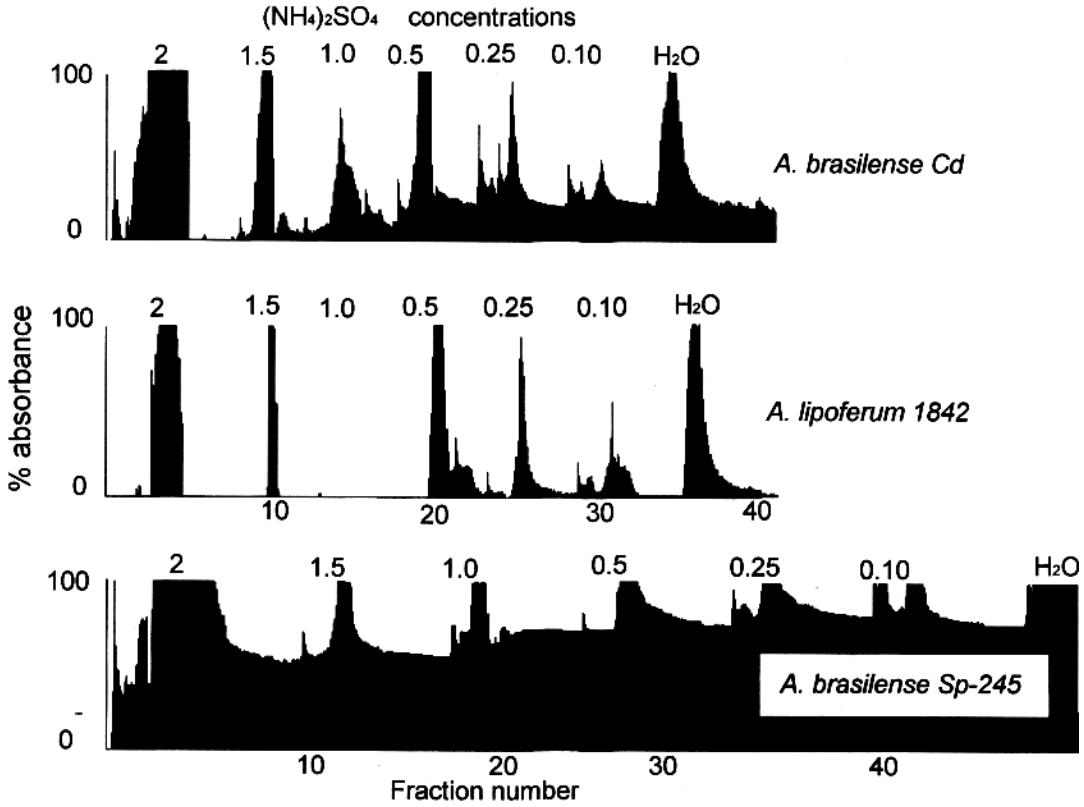

Fig. 4. Computer-enhanced image of hydrophobic chromatography of extracted surface proteins of *A. brasilense* Cd, *A. lipoferum* 1842 and *A. brasilense* Sp-245.

4. Discussion

Azospirillum spp. have two different modes of attachment to wheat roots, adsorption and anchoring. Adsorption and anchoring are probably different phenomena [13,21]. This study deals only with adsorption variables designed to mimic environmental factors. CSC and CSH properties of plant growth-promoting rhizobacteria (PGPR) such as *Azospirillum*, and their possible involvement in the first step of the adsorption process to roots might have an environmental significance. Physicochemical characteristics of the cell surface may play a role in the nonspecific and specific adhesion of bacteria to plant cell surfaces [33]. Bacterial surface hydrophobicity can be used to estimate the overall adhesion potential of bacteria to soil particles [34]. Adsorption data may have a practical use in assisting the selection of the most adequate PGPR strains for bacterial inoculants in future agriculture.

Bacterial cell-surface properties are, in general, affected by the culture medium composition, growth conditions, and culture age [26,35], and influence the attachment of marine bacteria to polystyrene [36]. Other studies showed that when *A. brasilense* Cd was evaluated in a two phase water:*n*-hexane partitioning system, it showed no hydrophobicity. But in the presence of cetrimide, its CSH increased [25]. Recently, cell-surface protein concentration and cell-surface hydrophobicity were increased in *A. brasilense* and were correlated with an increase of cell adhesiveness [26]. Our data propose that the CSC and CSH of the 10 *Azospirillum* strains tested are culture medium dependent, and bacterial strain dependent, similar to the human pathogen *Candida* and marine bacterium *Vibrio* [37-39]. Culture conditions

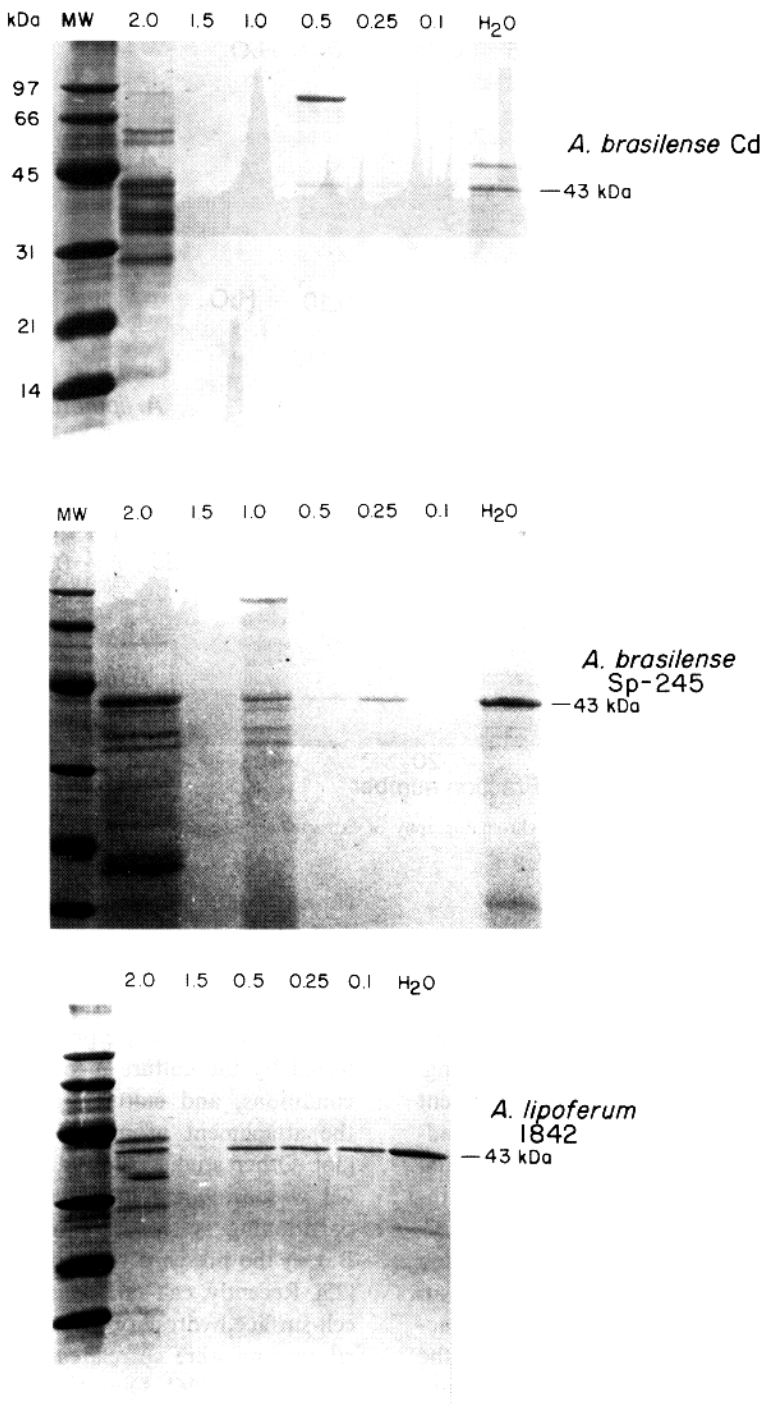

Fig. 5. SDS-PAGE of the surface proteins and fractions obtained from hydrophobic chromatography eluted with a step gradient of decreasing $(NH_4)_2SO_4$ concentration (1.5, 1.0, 0.5, 0.25, 0.1 M, and distilled H_2O) of (A) *A. brasilense* Cd, (B) *A. brasilense* Sp-245 and (C) *A. lipoferum* 1842. Molecular mass markers are in lane I.

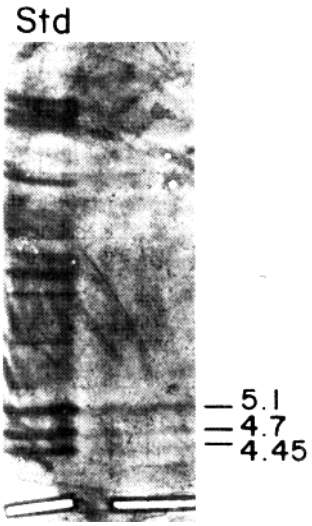

Fig. 6. Isoelectric focusing of the purified hydrophobic protein of *A. lipoferum* 1842. Std, protein standards.

of *A. brasilense* Cd and root treatment with chemicals, nutrients, chemoattractants, enzymes, or various environmental conditions like microaerophilic growth, greatly affect the attachment of the bacteria to root surfaces. High levels of adsorbed bacteria were recorded when the bacteria were grown in the presence of KNO_3 or when inoculated under hydroponic conditions [10]. Our current study indicates that environmental variables may change the CSC and CSH, as many other culture conditions did, and so facilitate attachment. However, this assumption needs to be confirmed with plants.

Azospirillum spp. produce, in solid, semisolid, or static liquid media, a lateral flagellum and many fimbria-like lateral structures [18]. These structures sometimes have a hydrophobic character [40], which may explain the increased value of CSH we found when *Azospirillum*. spp. were grown on solid media. Fimbrias, as in *Klebsiella* spp., have been previously reported to be responsible for the adhesion to plant roots [41] and to other tissues, like the adhesion of *P. aeruginosa* to human respiratory epithelial cells and of *E. coli* to intestinal mucin [40]. Nonetheless, in the PGPR *P. putida* and *P. fluorescens*, the role of cell-surface properties was unimportant for the adhesion process to potato roots [42]. In liquid medium, *Azospirillum* cells usually produce only the polar flagellum and not the lateral flagella [43,44]. The absence of the numerous lateral flagella in liquid media, a fact that may reduce the hydrophobic surface area of the bacterium cell, may explain the low CSC and CSH found in this study when the cells were grown in liquid media.

Our study showed that the genus *Azospirillum* has generally a low CSH value compared to other bacteria such as *Vibrio cholerae* [37]. This may show the relatively low impact of cell-wall hydrophobicity and charge on the initial attachment by the bacteria. The CSH variability in our study may be the outcome of the expression of varying amounts and types of polysaccharides, a variable number of fimbria per strain, the amount of capsular material [21,45], and cell pleomorphism [46]. All of these depend on the medium composition, growth stage, and agitation of the culture during growth [18,47].

Azospirillum spp. are being applied to plants in either solid or liquid inoculants [1]. This study proposes that the inoculant form may affect the initial adhesiveness of the bacteria, being more hydrophobic and with greater charge at solid surfaces. With increased attachment of bacteria to the inoculant surface, this inoculant type may not release the bacteria as readily as liquid inoculant. Future inoculant research needs to be focused on this point. Although it is known that *Azospirillum* attached quickly to surfaces [22], the variables controlling this attachment, evaluated in this study, were overlooked. Knowledge of the charge and hydrophobicity of a particular strain to be used as inoculant might give a preliminary inductive clue about the prospects of success of the strain as inoculant.

Several bacterial cell-associated proteins have been described as involved in cell adhesion of human bacterial pathogens [48] and other strains to plant roots, like *Klebsiella* sp. to *Poa pratensis* [41,49], *Rhizobium leguminosarum* to pea roots [50,51], and *Rahnella aquatilis* to wheat roots [52]. Cell-wall proteins of *A. brasilense*, which act as a receptors for siderophores [53], and the cell-wall protein involved in adhesion of *Rahnella aquatilis* to wheat roots [52] have been isolated. From the data presented in this study, it is premature to evaluate the role of our isolated hydrophobic protein from *A. lipoferum* 1842 in the overall adhesion process of *Azospirillum* to surfaces. The isolation of a cell-associated hydrophobic protein from *Azospirillum* spp. does not rule out the

possibility that other cell-wall components are involved in the overall adhesion process. It is likely that the general mechanism of attachment to roots by fibrillar material and massive aggregation of bacterial cells into flocs is also related to the polysaccharide content of the cell surface [19,21,45,54,55].

Given the data collected so far on the cell-wall properties of *Azospirillum* spp., one can conclude that the variability encountered in CSC and CSH originates from profoundly different cell-wall characteristics of the different strains. Although it appears that these parameters varied accidentally, a different view might conclude that there might be a practical way (albeit different for each strain) of influencing hydrophobicity and charge of the cell wall, and by this directly affecting attachment. This hypothesis is currently under study.

In summary, our study demonstrates that external physicochemical factors (resembling environmental variables) can change the cell-surface hydrophobicity and the cell-surface charge of 10 *Azospirillum* strains. As such they might have an impact on the first attachment process of these bacteria to surfaces.

Acknowledgments

We thank Mr. Ariel Cruz for excellent technical assistance and Dr. Ellis Glazier for constructive English corrections. Yoav Bashan participated in this study in memory of the late Mr. Avner Bashan from Israel. This study was supported by two grants (#3541-A and #87149) from the Consejo Nacional de Ciencia y Tecnologia (CONACyT), Mexico.

References

[1] Bashan, Y. and Holguin, G. (1997) *Azospirillum*-plant relationships: environmental and physiological advances (1990-1996). Can. J. Microbiol. 43, 103-121.

[2] Okon, Y. and Labandera-Gonzalez, C.A. (1994) Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol. Biochem. 26, 1591-1601.

[3] Parke, J.L.(1991) Root colonization by indigenous and introduced microorganisms. In: The Rhizosphere and Plant Growth. (Keister, D.L. and Cregan, P.B., Eds.), pp. 33-42. Kluwer, Dordrecht.

[4] Bashan, Y., Levanony,H. and Klein, E.(1986) Evidence for a weak active external adsorption of *Azospirillum brasilense* Cd to wheat roots. J. Gen. Microbiol. 132, 3069-3073.

[5] Bashan, Y. and Holguin, G. (1994) Root-to-root travel of the beneficial bacterium *Azospirillum brasilense*. Appl. Environ. Microbiol. 60, 2120-2131.

[6] Bashan, Y, and Holguin, G. (1995) Inter-root movement of *Azospirillum brasilense* and subsequent root colonization of crop and weed seedlings growing in soil. Microb. Ecol. 29, 269-281.

[7] Bashan, Y, and Levanony, H. (1987) Horizontal and vertical movements of *Azospirillum brasilense* Cd in the soil and along the rhizosphere of wheat and weeds in controlled and field environments. J. Gen. Microbiol. 133, 3473-3480.

[8] Assmus, B., Hutzler, P., Kirchhof, G., Amann, R., Lawrence, J.R. and Hartmann, A. (1995) In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. Appl. Environ. Microbiol. 61, 1013-1019.

[9] Bashan, Y. and Holguin, G. (1993) Anchoring of *Azospirillum brasilense* to hydrophobic polystyrene and wheat roots. J. Gen. Microbiol. 139, 379-385.

[10] Bashan, Y. and Levanony, H. (1989) Factors affecting adsorption of *Azospirillum brasilense* Cd to roots hairs as compared with root surface of wheat. Can. J. Microbiol. 35, 936-944.

[11] Bilal, R., Rasul, G., Arshad, M. and Malik, K.A. (1993) Attachment, colonization and proliferation of *Azospirillum brasilense* and *Enterobacter* spp. on root surface of grasses. World J. Microbiol. Biotechnol. 9, 63-69.

[12] Croes, C., Moens, S., Van Bastelaere, E., Vanderleyden, J. and Michiels, K. (1993) The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. J. Gen. Microbiol. 139, 1261-1267.

[l3] Michiels, K.W., Croes, C.L. and Vanderleyden, J. (1991) Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. J. Gen. Microbiol. 137, 2241-2246.

[14] Bashan, Y. and Levanony, H. (1988) Adsorption of the rhizosphere bacterium *Azospirillum brasilense* Cd to soil, sand and peat particles. J. Gen. Microbiol. 134, 1811-1820.

[15] Bashan, Y. and Levanony, H. (1988) Active attachment of *Azospirillum brasilense* Cd to quartz sand and to light-textured soil by protein bridging. J. Gen. Microbiol. 134, 2269-2279.

[16] Eyers, M., Vanderleyden, J. and Van Gool, A. (1988) Attachment of *Azospirillum* to isolated plant cells. ELMS Microbiol. Lett. 49, 435-39.

[17] Del-Gallo, M., Negi, E. and Neyra, C. A. (1989) Calcofluor and lectin binding exocellular polysaccharides of *Azospirillum brasilense* and *Azospirillum lipoferum*. J. Bacteriol. 171, 3504-3510.

[18] Madi, L., Kessel, M., Sadovnik, L. and Items, Y. (1988) Electron microscopic studies of aggregation and pellicle formation in *Azospirillum* spp. Plant Soil 109, 115-121.

[19] Neyra, C.A., Atkinson, A. and Olubayi, O. (1995) Coaggregation of *Azospirillum* with other bacteria: basis for functional diversity. In: *Azospirillum* VI and Related Microorganisms (Fendrik, T., Del-Gallo, M., Vanderleyden, J. and de Zamaroczy, M., Eds.), pp. 429-439. Springer, Heidelberg.

[20]Marshall, K.C. (1986) Adsorption and adhesion processes in microbial growth at interfaces. Adv. Colloid Interface Sci. 25, 59-86.
[21]Michiels, K., Verreth, C. and Vanderleyden, J. (1990) *Azospirillum lipoferum* and *Azospirillum brasilense* surface polysaccharide mutants that are affected in flocculation. J Appl Bacteriol. 69, 705-711.
[22]Gafny, R., Okon, Y. and Kapulnik, Y. (1986) Adsorption of *Azospirillum brasilense* to corn roots. Soil Biol. Biochem. 18, 69-75.
[23]Ascencio, F., Johansson, G. and Wadström, T. (1995) Cellsurface charge and cell-surface hydrophobicity of collagenbinding *Aeromonas* and *Vibrio* strains. Arch. Microbiol. 164, 223-230.
[24]Rosenberg, M. and Doyle, R.J. (1990) Microbial cell-surface hydrophobicity: history, measurements, and significance. In: Microbial Cell-Surface Hydrophobicity (Rosenberg, M, and Doyle, R.J., Eds.), pp. 1-37. American Society for Microbiology, Washington, DC.
[25]Arunakumari, A., Lamm, R.B. and Neyra-Estens, C.A. (1992) Changes in cell-surface properties of azospirilla in relation to cell pleomorphism and aggregation. Symbiosis 13, 291-305.
[26]Dufrêne, Y.F. and Rouxhet, P.G. (1996) Surface composition, surface properties, and adhesiveness of *Azospirillum brasilense* - Variation during growth. Can. J. Microbiol. 42, 548-556.
[27]Bashan, Y., Holguin, G. and Lifshitz, R. (1993) Isolation and Characterization of plant growth-promoting rhizobacteria. In: Methods in Plant Molecular Biology and Biotechnology (Glick, B.R, and Thompson, J.E., Eds.), pp. 331-345. CRC Press, Boca Raton, FL.
[28]Okon, Y., Albrech, S.L. and Burris, R.H. (1977) Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl. Environ. Microbiol. 33, 85-88.
[29]Johansson, G. (1974) Partition of proteins and micro-organisms in aqueous biphasic systems. Mol. Cell. Biochem. 30, 169-180.
[30]Paulsson, M., Liang, O.D., Ascencio, F. and Wadström, T. (1992) Vitronectin-binding surface proteins of *Staphylococcus aureus*. Zbl. Bakt. 277, 54-64.
[31]Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.
[32]Pharmacia Fine Chemicals (1982) Isoelectric Focusing. Principles and Methods, pp. 454. Llungföretogen, Örebro.
[33]Smit, G. and Stacey, G. (1990) Adhesion of bacteria to plant cells: role of specific interactions versus hydrophobicity. In: Microbial Cell-Surface Hydrophobicity (Rosenberg, M. and Doyle, R.J., Eds,), pp. 179-209. American Society for Microbiology, Washington, DC.
[34]Stenström, T.A. (1989) Bacterial hydrophobicity, an overall parameter for the measurement of adhesion potential to soil particles. Appl. Environ. Microbiol. 55, 142-147.
[35]McEldowney, S. and Fletcher, M. (1986) Effect of growth conditions and surface characteristics of the aquatic bacteria on their attachment to solid surfaces. J. Gen. Microbiol. 132, 513-523.
[36]Fletcher, M. (1977) The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. 23, 1-6.
[37]Faris, A., Lindahl, M. and Wadström, T. (1982) High surface hydrophobicity of hemagglutinating *Vibrio cholera* and other Vibrios Curt. Microbiol. 7, 357-362.
[38]Glee, P.M., Sundstrom, P. and Hazen, K.C. (1995) Expression of surface hydrophobic proteins by *Candida albicans* in vivo. Infect. Immun. 63, 1373-1379.
[39]Hasen, K.C., Plotkin, B.J. and Klimas, p.m. (1986) Influence of growth conditions on cell-surface hydrophobicity of *Can-dida globrata*. Infect. Immun. 54, 269-271.
[40]Irvin, R.T. (1990) Hydrophobicity of proteins and bacterial fimbriae. In: Microbial Cell-Surface Hydrophobicity (Rosenberg, M. and Doyle, R.J., Eds.), pp. 137-177. American Society for Microbiology, Washington, DC.
[41]Korhonen, T.K., Tarkka, E., Ranta, H, and Haahtela, K. (1983) Type 3 fimbriae of *Klebsiella* sp.: molecular characterization and role in bacterial adhesion to plant roots. J. Bacteriol. 155, 860-865.
[42]Weger, L., Van Loosdrecht, M.C.M., Klaassen, H.E. and Lugtenberg, B.J.J. (1989) Mutational changes in physicochemical cell-surface properties of plant-growth-stimulating *Pseudomonas* spp. do not influence the attachment properties of the cells. J. Bacteriol. 171, 2756-2761.
[43]Moens, S., Michiels, K., Keijers, V., Van Leuven, F. and Vanderleyden, J. (1995) Cloning, sequencing, and phenotypic analysis of laf 1, encoding the flagellin of the lateral flagella of *Azospirillum brasilense* Sp7. J. Bacteriol. 177, 5419-5426.
[44]Zhulin, LB. and Armitage, J.P. (1993) Motility, chemokinesis, and methylation-independent chemotaxis in *Azospirillum brasilense*. J. Bacteriol. 175, 952-958.
[45]Del Gallo, M. and Haegi, A. (1990) Characterization and quantification of exocellular polysaccharides in *Azospirillum brasilense* and *Azospirillum lipoferum*. Symbiosis 9, 155-161.
[46]Bashan, Y., Levanony, H. and Whitmoyer, R.E. (1991) Root surface colonization of non-cereal crop plants by pleomorphic *Azospirillum brasilense* Cd. J. Gen. Microbiol. 137, 187-196.
[47]Lamm, R.B. and Neyra, C.A. (1981) Characterization and cyst production of azospirilla isolated from selected grasses growing in New Jersey and New York. Can. J. Microbiol. 27, 1320-1325.
[48]Owen, P. (1988) Outer membrane proteins: their role in virulence. In: Immunochemical and Molecular Genetic Analysis of Bacterial Pathogens (Owen, P. and Foster, T.J., Eds.), pp. 273. Elsevier, New York.
[49] Haahtela, K.E., Tarkka, E. and Korohonen, T.K. (1985) Type 1 fimbria-mediated adhesion of enteric bacteria to grass roots. Appl. Environ. Microbiol. 49, 1182-1185.
[50]Smit, G., Kijne, J.W. and Lugtenberg, B. J. J. (1989a) Roles of flagella, lipopolysaccharide, and a Ca^{2+}-dependent cell-surface protein in attachment of Rhizobium leguminosarum biovar *viciae* to pea root hair tips. J. Bacteriol. 171, 569-572.
[51]Smit, G., Logman, T.J.J., Boerrigter, M.E.T.I., Kijne, J.W. and Lugtenberg, B.J.J. (1989b) Purification and partial characterization of the *Rhizobium leguminosarum* biovar *viciae* Ca^{2+}-dependent adhesin, which mediates the first step in at-

tachment of cells of the family Rhizobiaceae to plant root hair tips. J. Bacteriol. 171, 4054-4062.
[52]Achouak, W., De Mot, R, and Heulin, T. (1995) Purification and partial characterization of an outer membrane protein involved in the adhesion of *Rahnella aquatilis* to wheat roots. FEMS Microbiol. Ecol. 16, 19-24.
[53]Bachhawat, A.K. and Ghosh, S. (1987) Isolation and characterization of the outer membrane proteins of *Azospirillum brasilense*. J. Gen. Microbiol. 133, 1751-1758.
[54]Levanony, H., Bashan, Y., Romano, B. and Klein, E. (1989) Ultrastructural localization and identification of *Azospirillum brasilense* Cd on and within wheat root by immuno-gold labeling. Plant Soil 117, 207-218.
[55]Zaady, E. and Okon, Y. (1990) Cultural conditions affecting *Azospirillum brasilense* cell aggregation and adsorption to maize roots. Soil Biol. Biochem. 22, 1103-1107.